L-ASPARAGINASE ENZYME PRODUCING HALOPHILIC BACTERIA ISOLATED FROM SAMBHAR SALT LAKE EXHIBITING ANTICANCER ACTIVITY

RESEARCH PLAN

FOR REGISTRATION IN DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this Research Proposal entitled **"L-Asparaginase Enzyme Producing Halophilic Bacteria Isolated from Sambhar Salt Lake Exhibiting Anticancer Activity"** by **Chesta Saini,** submitted in fulfilment of the requirement for registration in Ph.D. program in **Department of Microbiology** under Faculty of Sciences of JECRC University, Jaipur, during the academic year 2022, is a bonafide record of work carried out under my guidance and supervision.

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INTRODUCTION

Microorganisms can survive in extreme climates and are known for producing useful compounds. In halophilic micro-organisms bacteria, archaea and eukaryote are included and that have grown in a saline environment and it is a major characteristic in halophiles, also known as "salt-loving". Natural microbial communities in hypersaline habitats are made up of halophilic bacteria (Oren, 2008), which are found globally. Halophiles need Na^+ for metabolising and development. Halophiles are divided into three categories based on their NaCl requirement for growth: low concentration (1–3%) called as slight, medium concentration (3–15%) called as moderate, and high concentration (15–30%) called as extreme. Unlike halotolerant micro-organisms, obligatory halophilic micro-organisms need more than 3% NaCl concentration or higher than salt water salinity with almost 3.5% NaCl concentration (Corral et al., 2020). Some bacteria secreted compounds, such as enzymes, can inhibit the cancer growth (Song et al., 2018).

Many therapeutic enzymes have been utilised in cancer therapy such as L-arginase, L-glutaminase, L-tyrosinase, L-asparaginase, L-methioninase, galactosidase and glucosidase (Farahmand et al., 2018). Animals, fungi, plants and bacteria are the source of these enzymes (Reshma, 2019). A probable resource for the synthesis of enzymes with different immunological characteristics could be halotolerant and halophilic bacteria accompanied by unique enzymatically properties. In the production of large quantities of these enzymes then the microbial sources are the great resource. L-glutaminase and L-asparaginase are effectively used in treatment of a person which is suffering with acute lymphoblastic leukaemia (ALL) and cancer cell. The strain that produced extreme L-asparaginase didn't produce the vital medicinal enzyme L-glutaminase (Shirazian et al., 2016). On the other hand, one of the most fundamental concerns in modern biology is the onset of cancer (Frank, 2018).

Cancer is a complicated disease that is caused by various interactions between genes and the environment and it is one of the world's current leading causes of death (Fearon et al., 2001; Mattox, 2017). Cancer is a severe global problem that causing more than 80 lakh people deaths every year. IARC (International Agency for Research on Cancer) stated that, cancer is leading cause for 76 lakhs dying globally and 127 lakh new patient are expected every year. Developing countries have the highest cancer risk, according to a survey 63% of cancer

related deaths were recorded only from developing countries (Abbas and Rehman, 2018). By 2030, it is estimated that 170 lakh people would die (Thun et al., 2010). Various cancers appear differently in different people, and cancers can vary within a given patient due to differences in the clonality of cancer cells and/or the surrounding micro environment (Parsons, 2018). Furthermore, some malignancies originate as a result of chronic inflammation, while others can subvert and/or co-opt an immune response as part of their progression and metastasis (Hegde et al., 2020). In recent decades, medical and pharmaceutical research has focused on bacteria's therapeutic potential. Enzyme therapy is one of these strategies that results in a more straight forward and targeted cancer treatment (Gonzalez, 1969). The fundamental idea behind enzyme therapy methods is to use specific enzyme that can deplete the amount of a specific form of amino acid that is required for malignant cell expansion but not for traditional cell survival (Zolfaghar et al., 2019). The first reporting on L-asparaginase enzyme potential as treatment for cancer was in 1953 (Kidd, 1953).

Several investigation studies have found that the bacteria secreted several medicinal probable compounds which used in treatment of cancer cell lines (Song et al., 2018). Human cancer derived cell lines are useful models for researching cancer biology and evaluating the therapeutic efficacy of anticancer medicines in the laboratories (Sharma et al., 2010). *Helicobacter pylori* strains that has been linked to gastritis cancer (Correa et al., 1990). In 2019 in India, a *Piscibacillus* sp. strain C12A1 obtained by Sambhar Salt Lake showed anticancer activity against the breast cancer MDA-MB-231 cell line (Neelam et al., 2019). Current study shows interest in outcome which suggest that in cancer treatment halophilic bacteria might be also efficient agent and can be perfect placement method for the selected cancer treatment.

L-asparaginase enzyme from mesophilic bacteria such as *Erwinia chrysanthemi* and *E. coli* has been utilized as an antineoplastic agent with certain limitations because it can cause some side effects when used in long-term which lead to hypersensitivity/allergic reactions and liver failure too, etc. L-asparaginase enzyme producing by halophilic bacteria have unique immunological characteristics which can used for allergic/hypersensitive persons (Ebrahiminezhad et al., 2011). That’s why, we require a new L-asparaginase enzyme with advance immunological properties. At present, only a few numbers of studies showed the L-asparaginase enzyme activity against cancer cell line.

REVIEW OF LITERATURE

Acinetobacter baumannii ZAS1 produced L-asparaginase enzyme and showed a significant production level of L-asparaginase with 45.59 ± 0.36 U/ml (Abhini et al., 2022). *Bacillus halotolerans* OHEM18, a novel bacterial strain, was identified to produce extracellular L-asparaginase in the present study. L-asparaginase was purified to 3.84-fold by ammonium sulphate precipitation on a QFF column, with a specific activity of 215.33 U/mg and a molecular mass of 41.5 kDa. The maximum enzyme activity was determined at pH 8.2 and 40 °C. The values for Km and Vmax were found to be 0.0047 M and 92.74 μM/ml/min. On vero cells, a cytotoxicity test revealed that L-asparaginase were safe, with selectivity against for leukaemia, breast cancer, and hepatoma cells. With an IC_{50} of 10290 gl^{-1} and a selectivity index of 30.61, NFS-60 cells were the most sensitive tumor cells to L-asparaginase and L-asparaginase showed antioxidant activity against both ABTS and DPPH radicals with IC_{50} values of 64.07 and 177.1 gl^{-1} (El-Fakharany et al., 2022).

Improve the production of L-asparaginase, a biologically important molecule, by the halotolerant *Bacillus licheniformis* PPD37 strain by bioengineering medium components through data and process centric approaches. Statistical techniques such as response surface methodology (RSM) – process centric and artificial neural network (ANN) – data centric approaches were used in the optimisation investigation. The production increased by roughly 6 times from 2.86 U/ml to 17.089 U/ml, compared to the unoptimized L-asparaginase production. Large-scale L-asparaginase production, as this is the first report to the author's best knowledge on the construction of an inference system employing RSM and ANN models for enhanced L-asparaginase synthesis using a halotolerant bacterium (Patel et al., 2022).

The significant toxicity and adverse effects of today's FDA-approved L-asparaginase, which are especially difficult in adult patients, are one of the key factors restricting their use in solid tumors. The development of improved L-asparaginase molecules offers the possibility of overcoming these undesirable side effects. Another problem in expanding the use of L-asparaginase is figuring out how cells can counteract the drug's pharmacological effects and identifying L-asparaginase resistance mechanisms. With a focus on both haematological and solid malignancies, this study highlights recent insights into L-asparaginase adverse effects, resistance mechanisms, and how

novel L-asparaginase variations and medication combinations can increase its therapeutic utility (Van Trimpont et al., 2022).

L-asparaginase enzyme producing bacteria were screened and identified as *B. altitudinis* KB1from a marine source in coastal areas of Kerala, India and enzyme assay showed a significant production level of L-asparaginase with 2.537 U/ml (Hadi et al., 2021).

L-asparaginase efficiency determined by kinetics and substrate selectivity, which differ from one source to another. The morphological and genotypic characterization of bacteria with L-asparaginase activity isolated from Maras, Pilluana, and Chilca salterns and 24 bacteria tested were divided into three groups: 38% Gram-negative bacilli, 54% Gram-positive bacilli and 8% Gram-positive cocci. In 14 of the 24 bacteria producing L-asparaginase in solid media and the Nessler technique was used to evaluate enzymatic activity in submerged cultures. By partial sequencing of the 16S ribosomal gene, the CH11, M62, M64, M68, and P19 strains were identified as *Bacillus* sp. They had greater L-asparaginase activity and instability due to the presence of proteases. Bacteria from saline environments could be used to produce L-asparaginase, which could be used as a therapeutic agent (Montes et al., 2021).

Isolates: PGF4 exhibited L-asparaginase enzyme activity and L-arginase enzyme activity, and BVUC_01, BVUCh_02, *Staphylococcus aureus, Aspergillus versicolor*, *Salmonella typhi*, *Bacillus velezensis*, *Bacillus subtilis*, *Bacillus licheniformis* and PGF2 yielded L-asparaginase enzyme, L-arginase enzyme and L-glutaminase enzyme. Only L-arginase was synthesized by *P. aeruginosa*, *C. freyschussii* and *B. megaterium*. In the presence of medium to contain 10% (w/v) NaCl, *A. versicolor* showed L-asparaginase enzyme, L-arginase enzyme and L-glutaminase enzyme (Parmar et al., 2021).

A total of 23 strains were obtained, 17 strains showed the production of extracellular L-asparaginases enzyme, and *Bacillus* strain which are identified as gram +ve bacilli produced the maximum L-asparaginase enzyme activity was 2 U/ml (Patil, 2021).

In Urmia Lake, 2 strains *H. elongate* and *B. aryabhattai* produced L-asparaginase enzyme, and not exhibited any negative impact on persons which deals with cancer (Moghadam and Khadivi Derakhshan, 2019). *Bacillus velezensis* isolated from marine sediments and the anticancer activity of the glutaminase-free L-asparaginase enzyme against breast adenocarcinoma cell lines

demonstrated significant activity toward MDA-MB-231 cells when compared with MCF-7 cells with IC_{50} values of 0.0126 ± 0.0012 gl^{-1} and 0.0173 ± 0.0028 gl^{-1}, respectively (Mostafa et al., 2019).

Out of 110 halophilic and halotolerant isolates, 29 L-asparaginase, 2 L-arginase and 4 L-glutaminase were produced. Enzymes were produced in the stationary phase of growth and the maximum enzyme activity was experienced in *Vibrio* sp. strain GBPx3 for L-asparaginase at 1.0 U/ml, *Rhodococcus* sp. strain R2S25 for L-glutaminase at 0.6 U/ml and *Planococcus* sp. strain GAAy3 for L-arginase at 3.1 U/ml (Zolfaghar et al., 2019).

Pseudomonas fluorescens produced L-asparaginase enzyme, and accompanied by particular action of 26 U/mg, filtered L-asparaginase had a Km of 0.050 M and Vmax of 4.032 mol^{-1}min (Sindhu and Manonmani, 2018). L-asparaginase coding gene of *Halomonas elongate* was isolated, expressed in *E. Coli*, purified and characterized and enzyme showed cytotoxic effects against Jurkat and U937 cell lines with an IC_{50} of 2 and 1 U/ml and minor inhibitory effects on human umbilical vein endothelial cells were observed. No negative effect was detected on Chinese hamster ovary cell line and human erythrocytes cell line (Ghasemi et al., 2017).

Acinetobacter baumannii sp. gave higher L-asparaginase activity of 7.32 U/ml and highly antibiofilm of L-asparaginase recorded against *Klebsiella pneumoniae* followed by *Pseudomonas aeruginosa* with reduction of biofilm formation ratio to 32 and 41%, respectively (Muslim et al., 2016).

Total of 85 halotolerant and halophilic bacterial strains, 16 isolates (19%) exhibited L-asparaginase activity and 3 strains (3.5%) showed L-glutaminase activity and based on 16S rDNA sequence analysis, selected isolates for L-asparaginase and L-glutaminase production belong to the genus *Bacillus* and *Salicola* (Shirazian et al., 2016). *Pseudomonas putida* produced L-asparaginase at 0.255 gl^{-1} concentration and following 72 hours to exhibited higher toxicity effect on the cancer cell line (Hep-2 cell line) (Musim, 2015).

Twelve isolates involved 1 strain of *Zobellella* sp., 8 strains of *Bacillus* sp., 1 strain of *Oceanimonas* sp. and 2 strains of *Pseudomonas* sp. identified. L-asparaginase enzyme showed the highest (1.6 U/ml) and the lowest (0.20 U/ml) productivity were recorded for *Pseudomonas* sp. PG_01 and *Bacillus* sp. PG_13, respectively (Izadpanah Qeshmi et al., 2014).

Acinetobacter baumannii R7 produced L-asparaginase at 0.2 gl^{-1} concentration and exhibited greater toxicity effect on the human ovarian cancer cells (OAW-42 cell line) (59 and 51% survival) for 24 and 48 hrs, respectively (Muslim, 2014). *Bacillus* sp. and *Pseudomonas* sp. has the potential to produce red/pink coloured zone all over the colony and, showed positive result towards the L-asparaginase enzyme (Audipudi et al., 2013).

Three isolates were found to have ability to produce the L-asparaginase enzyme. For the purification of enzyme after ion exchange chromatography the Vmax values for all these three isolates were 83.3 μM/ml/min, 111.11 μM/ml/min and 90.90 μM/ml/min was achieved (Luhana et al., 2013).

Microorganisms that characterized belongs to the genus of *Pseudomonas aeruginosa, Bacillus* sp., *E. coli*, *Aeromonas* sp., *Proteus* sp. and *Serratia* sp. was found positive for L-asparaginase production on the basis of pink/red zone around the colony (Kamble et al., 2012).

Screened the halophilic bacteria which belongs to moderate group that produces extra and intracellular L-asparaginase enzyme, and *Bacillus* sp. BCCS 034 was producing the maximum extracellular L-asparaginase enzyme (1.64 U/ml supernatant) (Ebrahiminezhad et al., 2011).

Mesophilic bacteria produce the L-asparaginase enzyme, but some side effects such as liver failure, hypersensitivity, etc appear when used for a long-term. Therefore, there is a need for a new L-asparaginase enzyme with enhanced immunological properties. Halophilic bacteria have unique immunological properties that can be used for allergic persons. Moreover, some research has shown that the halophilic bacterial L-asparaginase enzyme may be able to prevent the spread of solid cancer cells and show anticancer activity against cancer cell line.

DESCRIPTION OF TOPIC

Halophilic micro-organisms have grown in a saline environment and also known as “salt-loving”. Some bacteria secreted compounds, such as enzymes, can inhibit the cancer growth (Song et al., 2018). However, it has been proven that numerous enzymes have anticancer characteristics. L-arginase enzyme, L-glutaminase enzyme and L-asparaginase enzyme from halophilic and halotolerant bacteria were selected on the basis of amino acid depletion in cancers (Zolfaghar et al., 2019). Cancer refers to a set of most commonly diagnosed in the world and

deadliest pathophysiological conditions that are among the conquered researcher's interest for decades (Diori Karidio and Sanlier, 2021). Targeting cancer amino acid biosynthesis as a plan of treating cancer and this method has gained more popularity in recent years (Dang et al., 2011; Koppenol et al., 2011). Several cancer cells have an auxotrophic response for some amino acids including L-arginine, L-asparagine and methionine (Rytting, 2010; Gao et al., 2019) and cancer growth is inhibited and impaired when certain amino acids are depleted by enzymes that breakdown amino acids (Wang et al., 2021). L-asparaginase enzyme was first bacterial enzyme to be authorised for use in cancer treatment. L-asparaginase hydrolyses L-asparagine and converted into ammonia and L-aspartic acid (Pieters et al., 2011) and a major component of the initial therapy for acute lymphoblastic leukaemia (ALL) is L-asparaginase. Due to the absence of L-asparagine synthetase enzyme in cancer cells, which limits the metabolic activities and causes the apoptosis/cell death (Brumano et al., 2019). Recently, it has been demonstrated that L-asparaginase enzyme may be capable to prevent the spread of solid cancer cells. So, the proposed study aims to isolation and characterization of potential halophilic bacterial diversity from the Sambhar Salt Lake, Rajasthan and try to find a L-asparaginase enzyme effect on cancer cell line by biological process using bacteria.

OBJECTIVES OF THE STUDY

- To collect the water sample from Sambhar Salt Lake, Jaipur and physicochemical characterization of sample
- To isolate and characterization of halophilic bacteria
- To screen out the L-asparaginase enzyme producing halophilic bacteria
- To identify potent L-asparaginase enzyme producing halophilic bacteria using 16S rRNA gene sequencing technique
- To study the L-asparaginase enzyme on cancer cell line (MDA-MB-231 or MCF-7)

STUDY AREA

The Sambhar Salt Lake- It is the nation's longest inland salt lake and it is situated in Sambhar Town, Jaipur district, Rajasthan, India, This site was located 80 km south-west of the Jaipur city at 26º58' North latitude and 75º 05' East longitudes. Surface area of the Salt Lake is 190 to 230 km^2 (73 to 89 sq mi) and maximum depth is 3 metre (9.8 ft) (Neelam et al., 2019).

METHODOLOGY

1. Sample collection and analysis of physicochemical characteristics of sampling sites –

Water sample will be collected with the help of sterile bottles from the water depths of 15 cm and store till for further investigation at 4 °C. A thermometer and pH strips will be used to examine the physiochemical parameters at the sampling location. Other physicochemical characteristics such as salinity, conductivity and TDS will be determined in the laboratory (Neelam et al., 2019).

2. Isolation, microscopic examination and characterization of bacteria-

a) Isolation-

Water sample will inoculate in culture broth and incubate at 37 °C for 3 to 7 days. Single colony will isolate by sample serial dilution method and spread plate method. Pure single colony of bacterial isolates will be obtained by repeatedly streaking and culture will maintain at 4 °C in 10% glycerol solution and sub-culture at 15 days intervals for further research study (Neelam et al., 2019).

b) Microscopic examination-

Morphological studies of bacterial colonies on the basis of their colour, size, shape, form and opacity will be examined under microscope. Bacterial colonies of different samples will be identifying by Gram's Staining method (Singh et al., 2019).

c) Growth optimization-

By changing the temperature in 5 °C increment between 20 to 60 °C, the impact of temperature on development and metabolism of bacterial isolates will be examining. By varying the pH levels from 5 to 11 at intervals of 1 pH units in order to analysis the impact of pH on the development of bacterial isolates will be examining, and also determine the impact of salinity on development of bacterial isolates will be examine at different salt concentration ranging to 0 to 30% (w/v) (Amoozegar et al., 2009).

d) Biochemical characterization-

Biochemical characterization of bacterial isolates will be performed by various biochemical tests like indole test, MR-VP test, citrate utilization, catalase, oxidase, urease, nitrate reduction and acid production from carbohydrates (Audipudi et al., 2013; Leon et al., 2014).

3. Screening of L-asparaginase enzyme potential isolates-

Isolates will be further observed for L-asparaginase activity. L-asparagine will be added into modified salt medium at a concentration of 5-10 gl^{-1} and glucose, agar supplemented with phenol red as pH indicator (Zolfaghar et al., 2019).

4. Extracellular L-asparaginase assay-

Broth culture will be removed by centrifugation and supernatant will be used to assay extracellular L-asparaginase activity. L-Asparaginase assay process relies on direct ammonia nesslerization. The final amount of Tris- HCl and enzyme solution will be 1500 µl. Reactions will begin by adding the solution of L-asparagine (500 µl), and it will be incubated in water bath at 37 °C for 20 minutes. Reactions will be stopped by adding of 500 µl of 15% (w/v) trichloroacetic acid, and distilled water will be used to adjust the solution to 4500 µl. The solution will be mixed with Nessler's reagent and it will be incubated for 15 minutes at room temperature. Utilizing a UV-visible spectrophotometer, the absorbance will be determined at a wavelength of 530 nm, after vortexing. Using different ammonia concentrations, a standard curve will be created (Ebrahiminezhad et al., 2011).

5. 16S rRNA sequencing-

a) Genomic DNA extraction-

Bacterial isolates genomic extraction of DNA using a modified form of the procedure. The bacterial isolates will be pellet out by centrifugation for 5 minutes and after that lysis solution 500 µl will be added, and isolates will be incubated in water bath for 5 minutes at 65 °C. Genomic DNA will be extracted in 3 phases with various ratios of organic solutions/solvents. Using equivalent volume of Chloroform and Isoamyl alcohol (24:1), and will then be carefully mixed through inversion 3 to 5 times and centrifugation for 5 minutes. Separating the aqueous layer in a

new tube and extracting it with an equivalent amount of Phenol, Chloroform and Isoamyl alcohol (25:24:1) and centrifuged for 5 minutes. Again, separating the aqueous layer in a new tube and extracting it with equivalent volume of Chloroform and Isoamyl alcohol (24:1) and centrifugation for 5 minutes. Propan-2-OL (iso-propyl alcohol) and a portion of the uppermost aqueous layer containing DNA will be poured in a sterile centrifuge tube, and the solution will then be carefully emulsified through inverting at room temperature and centrifugation for 3 minutes. Supernatant will be eliminated properly and DNA pellet will be mixed with 70 µl of NaCl solutions. After that 300 µl absolute cold ethanol will be poured and DNA will be precipitated and it will be incubated for 7 minutes at -20 °C and after centrifuged for 5 minutes, the ethanol will be removed carefully. Pellet will undergo single washing by 70% ethanol and centrifugation for 5 minutes. Pellet will be gently dissolving in 50 µl deionized water using a vortexing process, then held in storage at -80 °C for later use (Neelam et al., 2018).

b) 16S rRNA gene amplification and phylogenetic analysis-

The standard 27F forward and 1495R reverse primers will be used to amplify the 16S rRNA gene. The PCR product will be purified, the 16S rRNA gene's partial sequencing will be determined via amplified sequence of DNA by both strands. 1415 bp of 16S rRNA partial sequencing will be required for submission to GenBank.

Similar analysis will be performed using the RDP (https://rdpNaNe.msu.edu) and EzTaxon (http://www.eztaxon.org) databases "Identify" option. The related/closest sequencing will retrieve and % similarities will check afterward manually alignment by MEGA 7 and GenBank (NCBI database) will retrieve for additional sequences that will be utilized to construct phylogenetic tree. By NCBI nucleotide database will be used BLASTN (www.ncbi.nlm.nih.gov/blastn) to retrieve the 16S rRNA gene sequence for phylogenetic analyses and linked with the isolated bacterial sequence through MUSCLE programme develop in the MEGA 7. By manual sequences alignment editor, alignment will be purified and similar programme will be used in calculating the similar sequences of the related/closest sequencing consider every position of alignments involving gap. For phylogenetic studies, a huge set of sequences greater than 1262 base pair will be taking into considered and after, shorter subsets will be creating on the basis of sequences closeness through excluded isolates of unknown affiliation. Through ML technique (maximum likelihood method) will be used MEGA 7 accompanied by default setting to create a phylogenetic tree, employs the

K2+G+I concept of nucleotide substitution, this model will be the best fitted to presented datasets as stated by Bayesian Information Criterion (BIC). Bootstrapping of thousand replicates will be calculating to detect the confidence value for the edge of the highest/best achieving ML tree. Neighbour joining and maximum parsimony trees will also be built to utilized the MEGA 7, topologies of every trees will compare to verify the position of phylogenetic of isolates (Neelam et al., 2019).

6. L-asparaginase enzyme activity on cancer cell line-

a) MTT assay for cell viability –

The MTT method will be used to determine cell viability. The 96 well plate will be planted with cells of cancer approx. 5000 cells per well in DMEM (Dulbecco's modified eagle medium) supplemented to 1% penicillin/streptomycin, 10% fetal bovine serum and an environment of 5% carbon dioxide for 24 hours at 37 °C. Planting cells will be incubated individually with various concentrations of L-asparaginase enzyme containing extract for 24 hours. At the ending of incubation period, 10 µl of MTT solution will be poured to every well. At 37 °C, plates will be incubated for 2 hours and then medium will be entirely removed. Staining has been dissolved by Dimethyl sulfoxide (DMSO), and on an enzyme-linked immunosorbent assay (ELISA) plate reading, absorbance will be determined at a wavelength of 530 nm. In order to determine the efficient absorbance for the treatment wells relative to the respective control wells, MTT data sets for various cancer cell lines will be used to calculate the LC_{50} (Lethal concentration at which 50% of cells are killed) after 24 hours (Chowdhury et al., 2017).

b) Scratch assay-

This method will be used to determine the cell migration. In 35 mm tissue culturing plate, cells will be planted at a density of 200000 cells per plate and grown until they are 95% confluent. Cell scratch wound creating with a micropipette tip and washing with PBS (phosphate buffer solution) to eliminate the floating cells. In testing plates, cells will be individually treated for 24 hours with L-asparaginase enzyme containing extract, whereas control plates will get an equivalent amount of the relevant solvents. Under a Carl Zeiss inverted microscope, cells will be visible and cell

movement will be evaluated by measuring gap diameters over numerous fields, calculating the relative size/area with Image J, and statistical analysis (Chowdhury et al., 2017).

c) Colony formation and spheroid formation assay -

Performing the colony formation experiment, spreading the cells in 6 well plates at a seeded concentration of 2000 cells per well. Cells will either undergo L-asparaginase enzyme containing extract treatment or without treatment after one day. Cells will be rinsed with PBS (phosphate buffer solution), colonies fixed with methanol and stained using crystal violet after five days. The bright field inverted microscope will take photos of plates and colonies. Spheroid formation assay will be carried out using a mixture of 2X DMEM media (Dulbecco's modified eagle medium), 2% antibiotic penicillin/streptomycin and 20% FBS. 2X DMEM (Dulbecco's modified eagle medium) and agar at 1.2% will be combined in an equal amount to create the base layer. Cancer cells will be trypsinized, enumerated, plated with density of 1000 cells/plate. In equal amounts of agar at 0.6% and 2X DMEM (Dulbecco's modified eagle medium) containing cells will be added to create the top layer. Then, L-asparaginase enzyme containing extract with 300 µl of DMEM (Dulbecco's modified eagle medium) will be added on top layer of agar and incubated for 7-8 days. Every three days, replaced the top media with fresh media. Pictures of Spheroids will be captured and counted using microscope (Sharma et al., 2019).

7. Statistical analysis-

One-way ANOVA will be used to compare the mean values between the treatment and control groups and detect the important distinctions. Student t test (Graph pad prism) will be used to determine the statistical variations among treatment and control groups, and in this case, $p < 0.05$ will be regarded as statistical significance (Neelam et al., 2019).

EXPECTED OUTCOME

- Isolated halophilic bacteria could be an addition of Sambhar Salt Lake microbial diversity with industrial applications.
- Bacterial L-asparaginase enzyme could be useful in treatment of cancer cell lines. This study focused on the more effective and less toxic treatment regimens.
- This study will increase the scientific information for society and provide a platform for entrepreneurs to make their effort in the field of halophilic bacterial enzyme.

WORK PLAN

S. No.	**Research Activity**	**6**	**12**	**18**	**24**	**30**	**36**	*****
1.	Course Work							
2.	Literature Survey							
3.	Methodology Steps							
4.	Quarterly Reports							
5.	Communication of Papers							
6.	Preparation of Final Thesis							
7.	Submission of Thesis							

* As approved Extension

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